Distinct turnover in fungal communities along an alpine ridge-snowbed gradient

Master of Science Thesis

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Photos: Unni Vik and Marit F. M. Bjorbækmo

Preface

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Fang YAO

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ABSTRACT

The ridge-snowbed gradient is of high importance in alpine areas in structuring the vegetation cover. However, to what degree belowground fungal communities are affected by the gradient is much poorer understood. In this study, the fungal diversity and community composition associated with roots of the ectomycorrhizal plant *Bistorta vivipara* were studied along the ridge–snowbed gradient. Fifty root samples were collected in ten plots in an alpine area in central Norway and the fungal communities analyzed by 454 pyrosequencing analyses of tag encoded ITS1 amplicons. A distinct turnover in the fungal communities was found along the ridge-snowbed gradient, paralleled by changes in soil content of carbon, nitrogen and phosphorus. A large proportion (66%) of the detected 801 non-singleton OTUs belonged to Ascomycota, but basidiomycetes dominated quantitatively (i.e. number of reads). Numerous fungal OTUs, many with taxonomic affinity to Sebacinales, *Cortinarius* and *Meliniomyces,* showed distinct affinities to either ridge or snowbed plots, indicating habitat specialization. Although a turnover in fungal communities was observed, the diversity remained at the same level along the gradient.

Key words: *alpine, Bistorta vivipara, ectomycorrhiza fungal community composition, fungal richness, gradient, ridge and snowbed, root associated fungi, symbiosis*

INTRODUCTION

Background

The mycorrhizal symbiosis between fungi and plant roots are among the most ancient and prevalent symbiosis on Earth, and is crucial for the composition and functioning of terrestrial ecosystems. It is widely accepted that nutrient acquisition by the majority of land plants is mediated by mutualistic mycorrhizal fungi (Smith SE, 2008). Ectomycorrhiza (ECM) is most common in woody plants, and plays a key role for plant productivity in boreal and temperate forests, as nutrient availability in these ecosystems is usually low and most of the nutrients are present in organic form in litter and humus. Several recent studies have demonstrated a high diversity of ECM fungi in alpine and arctic ecosystems, as well (Bjorbaekmo et al., 2010; Blaalid et al., 2012)

Numerous factors and complex interactions influence the structure and composition of symbiotic fungal communities. Toljander et al. (2006) observed pronounced changes in ECM fungal communities along an environmental gradient in a boreal forest, and found that the fungal community composition was strongly correlated with various soil properties.
For example, the extractable NH_4 was found as a strong determinator of the ECM community, and further that moisture availability may influence ECM fungal distribution (Toljander et al., 2006). Similarly, fungal communities in soil exhibited high turnover in an alpine habitat, and were detected in association with different vegetation composition types and variable soil organic matter availabilities (Zinger et al., 2009; Zinger et al., 2011). pH has been argued to have variable influences on fungal communities. Rousk et al. (2010) found that pH had weak influence on fungal soil community in arable soil; contrasting a number of studies, such as Zinger et al. (2011) and Newbound et al. (2012), in which pH was shown to have a strong structuring effect on fungal communities. Furthermore, biotrophic interactions between ECM fungi and other organisms associated with the host plant have been found to influence the ECM fungal community (Pickles et al., 2012).

The 'model species' - *Bistorta vivipara*

Most ECM-forming plants are trees and shrubs, and due to their size, it is difficult to explore their entire root-associated fungal assemblages. However, a few herbs, including the circumpolar *Bistorta vivipara* (L.) Delarbre (syn. *Polygonum viviparum* L.) in the family

Polygonaceae, also form ECM. ECM in *B. vivipara* was first recognized by Hesselmann (1900) and has been confirmed in later studies (Read and Haselwandter, 1981; Lesica and Antibus, 1986; Eriksen, Bjureke, and Dhillion, 2002). *Bistorta vivipara* is a circumpolar species with a wide ecological amplitude that often occurs as a pioneer species in arctic and alpine environments (Dormann, Albon, and Woodin, 2002). It is a perennial polyploid, with high and variable chromosome numbers (2n=c.77-c.132; c.7*x*-12*x*) (Aiken, 1999 onwards; Vik et al., 2012).The plant produces bulbils (asexual propagules) in the lower part of the inflorescences and protandrous flowers in the upper part.

Bistorta. vivipara has been utilized as a model system for ectomycorrhizal research (Eriksen, Bjureke, and Dhillion, 2002; Blaalid et al., 2012; Vik et al., 2012). The small and condensed root system of B. vivipara allows the entire fungal community associated with each plant to be sampled and analyzed.

454 sequencing

Studying the ecological factors that underlie the dynamics of natural microbial communities remains a challenge, because of the high taxonomic diversity in such communities (Hawksworth, 2001). However, during the recent 20 years, the knowledge about ECM community ecology has increased dramatically due to the implementation of molecular DNA based methods (Pace, 1997; Horton and Bruns, 2001; Vandenkoornhuyse et al., 2002; O'Brien et al., 2005; Lindahl et al., 2007). These methods provide a way to survey biodiversity rapidly and comprehensively (Pace, 1997; Horton and Bruns, 2001). A challenge in ECM research has been the high number of replicates needed to capture and describe the complexity of soil microbial communities, which has made standard molecular methods less suited for analysis of such environmental samples. Recently, the application of high-throughput sequencing (HTS) technologies has initiated a new era, and enabled large scale analyses of complex fungal communities (Margulies et al., 2005) in soil (Buee et al., 2009) and associated with plant root (Kauserud et al., 2012). Among these new HTS technologies, high-throughput 454 DNA sequencing technology (Margulies et al., 2005) allows a much faster and more cost-effective sequencing strategy than traditional Sanger sequencing. High-throughput sequencing has in fact changed the entire approach by allowing “in depth” sequencing of virtually all targeted DNA molecules present in a given sample, providing potentially both qualitative and quantitative information. The power of 454 sequencing and its suitability for sequencing single DNA molecules within a mixture of molecules allow for the identification of the

dominant as well as rare variants of the sample. 454 sequencing provides via an emulsion PCR step an "instant cloning" of hundreds of thousands of molecules to be sequenced. Combined with the nuclear ribosomal internal transcribed spacer (ITS) region which has been adopted as a validated DNA barcode marker for fungal species identification (Seifert, 2009; Schoch et al., 2012), this provides a powerful tool for studying fungal diversity in environmental samples.

Aims

In this study, we investigated variation in fungal richness and community composition associated with *B. vivipara* roots in two different alpine vegetation types, i.e. ridge and snowbed, in central Norway by 454 sequencing of the internal transcribed spacer ITS. Ridge and snowbed are two major vegetation types in alpine habitats, which are characterized by different environmental and ecological properties and represent the extreme points of a gradient along which the vegetation is structured. Within snowbeds, the snow-cover is deep in winter and the vegetation melts out late in spring or summer. It is often more organic nutrient available underground but shorter growing season for plants in this vegetation type. At ridges, most snow is blown off by winds, and as soon as the temperature rises in spring, biological activities can increase significantly. The soil on the ridges is poorer in nutrient and the vegetation above ground has longer growing season (Fægri, 1967; Dahl, 1986) (Fig. 1) .

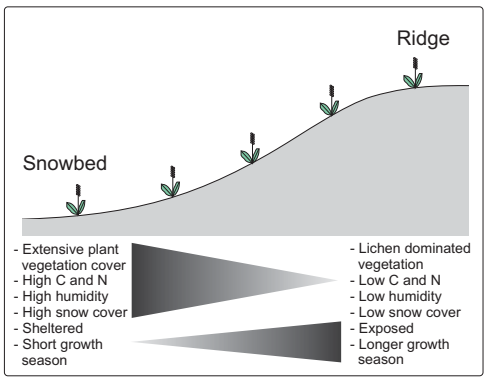

Fig. 1 Schematic figure of the ridge-snowbed gradient with environmental and ecological characteristics.

The main focus in this master project was to reveal whether *B. vivipara* forms root-associated symbiosis with different fungal partners across the ridge-snowbed gradient. More specifically, the aims were to test: (i) If there are differences in the composition and diversity of fungi

associated with *B. vivipara* across the different vegetation types (ridge and snowbed); (ii) If there is a spatial structure in the fungal communities across the sampling sites, independent of vegetation type; and (iii) If the soil nutrient concentration affects the diversity and composition of the root-associated fungi of *B. vivipara*.

MATERIALS AND METHODS

Sampling

The sampling area (60°35'N, 007°30'E, 1229 m to 1244 m above sea level) was located in the mid-alpine region at Finse, Norway (Fægri, 1967; Dahl, 1986). Samples were collected in two vegetation types; ridge and snowbed in July 2011 (Fig.2).

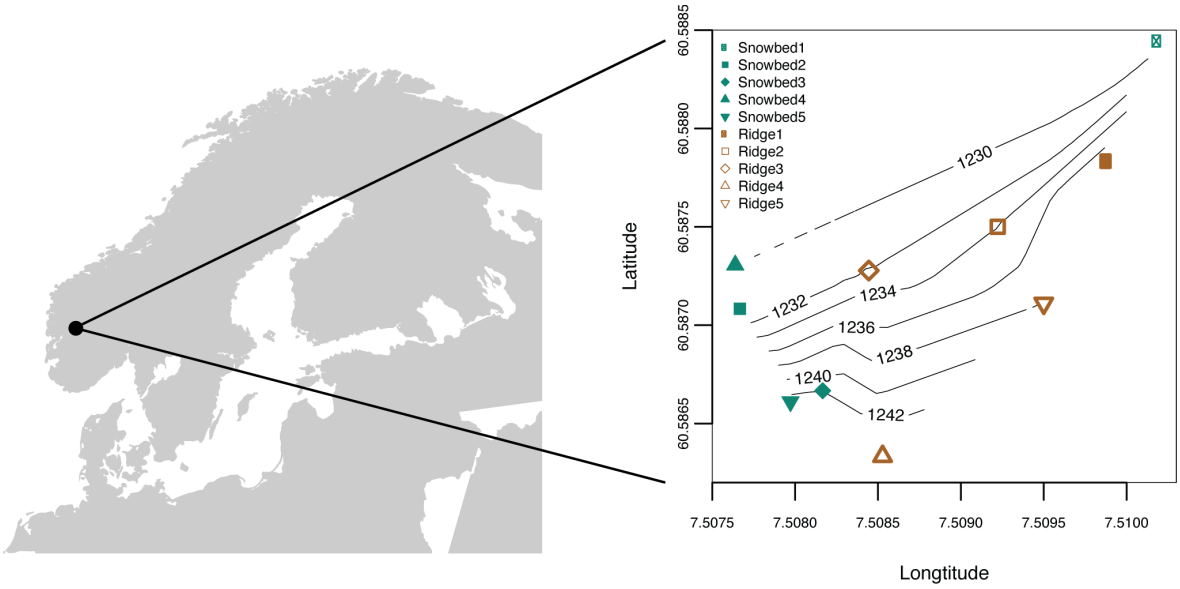

Fig.2 The sampling area for this study is located at Finse, Norway (60°35'N, 007°30'E), between 1229 m to 1244 m above sea level. The 10 plots, from which the 50 *Bistorta vivipara* root samples were collected are plotted with green symbols (snowbed) and brown symbols (ridge)

Within each vegetation type, five 1.5 m x 1.5m plots were placed randomly. Each plot was divided into a net grid of 15 cm x 15 cm squares (Fig. 3). All plant species found in each plot were recorded according to the point intercept method (Brathen and Hagberg, 2004). At each intercept in the grid, the species (alternatively stone or bare soil) first touched by a pin passed vertically through the vegetation were noted. All additional plant species present in the plot, but not recorded by this method, were noted. Plots from the ridge vegetation type were dominated by lichens (32%), while the snowbed plots were dominated by plant *Salix herbaceae* (42%) (Fig. S1). Five *B. vivipara* plants were collected from each plot, along with soil samples from below each plant (Fig. 3). Hence, a total of 50 plants and 50 soil samples were obtained.

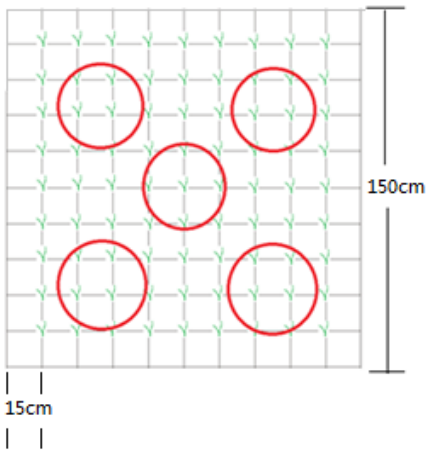

Fig.3 Schematic figure of a sample plot. The vegetation cover (frequency of plant species, alternatively stone and bare ground) was recorded by registering the plant species present at each intercept in the grid. Plant species present in the plot, but not registered by this method, were also noted. Five *Bistorta vivipara* were sampled, from the five circle areas: one plant from the center, and the rest four from the approximately middle points on the lines connecting the center and the four corners of the plot.

The soil samples were stored at -18°C within three hours after sampling. The whole root system of each plant sample was washed carefully free from soil and plant materials. The root threads were collected and placed into a 50 ml centrifuge tube (SARSTEDT, Nümbrecht, Gemany) and weighted. 1200 µl CTAB-lysis buffer (AppliChem GmbH, Ottoweg, Germany) was added to each tube before storing at -18°C. The length, width and height of each rhizome were recorded.

DNA extraction and 454 sequencing

After adding 10 beads and additional 5 ml 2% CTAB buffer with 2-mercaptoethanol (SIGMA CHEMIKAL CO, Steinheim, Germany), all 50 root system samples were crushed for 60 s at 4.0 m/s on a Fast Prep-24 beadbeater (M.P. Biomedicals, CA, USA). The samples were centrifuged at 1300 rpm in 30 seconds. Two samples (R1-73 and R2-63) were not well crushed and were additionally crushed for 60 s and centrifuged (1300 rpm in 30 s) again. From each tube, 20 ml of the aquatic phase was transferred to a new 20 ml centrifuge tube (SARSTEDT, Nümbrecht, Gemany) and frozen at -18°C. For DNA isolation, 600 µl of the crushed material was transferred to micro tube (SARSTEDT, Nümbrecht, Gemany). The samples were randomized before isolation to reduce methodological biases. For five randomly selected samples, two parallels were run as replicates to test for methodological biases (see

(Kauserud et al., 2012)). To extract DNA we used the Soil DNA isolation Kit (OMEGA Bio-tek, Norcross, GA, USA) according to the manufacturer's directions. The last step for collecting DNA by washing the filter was carried out twice, the first time producing DNA with a higher concentration, the second time with lower concentration. The DNA eluted in the second round was used downstream.

The ITS1 region was amplified using a nested PCR approach, as outlined in Blaalid et al. (2012). From each of the samples, 2 µl DNA was used as template for a nested PCR approach. In the first PCR, the fungal specific primer ITS1F and ITS4 were used to amplify the entire ITS region (White, 1990; Gardes and Bruns, 1993) using the following PCR program: denaturation for 30s at 98°C, followed by 30 cycles of denaturation for 10s at 98°C, annealing for 20 s at 50°C, and extension for 20 s at 70°C, then followed by a step of final extension for 7 min at 70°C, and cool-down at 10°C. Subsequently, ITS1 region was amplified using primers ITS2 and ITS5 (White, 1990) with 4 µl 20x diluted template from the first PCR. In the second PCR reaction samples were tagged in both ends by different pyrotags with a length of 10 bp (Table S1). The same PCR program was used. This PCR was run in triplicate and later pooled (Table S1). The resulting PCR products were cleaned up by using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, Madison, WI, USA) and normalized by using the SequalPrep™ Normalization Plate(96) kit (Invitrogen Inc., CA, USA). The PCR products were pooled according to tags in four tubes (Table S1), which were pyrosequenced on four lanes on half a plate (Roche GS FLX Titanium Series) at the Norwegian Sequencing Center (University of Oslo, Oslo, Norway, webpage: http://www.sequencing.uio.no).

Soil sample analyses

Soil samples were defrosted and sieved in sterile milliQ water to remove plant roots and debris and later dried. Concentration of phosphorus (P) was measured by using potassium persulfate ($K_2S_2O_8$). Samples were resolved in 10 ml 1% $K_2S_2O_8$ at 121°C in 30 min, and then run in a BRAN+LUEBBE autoanalyzer (Bran Luebbe, Norderstedt Germany) with the method of Multitest MT, no.G-297-03. The concentration of carbon (C) and nitrogen (N) was measured by a Thermo Finnigan EA 1112 Series Flash Elemental Analyzer (Thermo Scientific, Italy). The average concentrations of C were 9.9% for ridges and 32.59% for snowbeds, N with 0.6% and 2.2%, and P with 1.39% and 1.13%, respectively, for ridges and snowbeds.

Bioinformatics analyses

The sequence data was analysed using QIIME v. 1.5.0 (Caporaso et al., 2010). Reads with length <250 bp or >500 bp, an average Phred quality score <50 or any mismatch against the tags or ITS1 primers were removed. Denoiser v. 1.5.0 (Reeder and Knight, 2010) as implemented in QIIME v 1.5.0 (Caporaso et al., 2010) was used to denoise the retained 151642 reads. These denoised reads were clustered into operational taxonomic units (OTUs) using a 97% similarity threshold and the uclust algorithm as implemented in QIIME v. 1.5.0 (Caporaso et al., 2010). The most abundant sequence in each cluster was designated the representative sequence. The OTUs identified as putative chimeras based on the criteria of both (a) being identified as chimeric by the perseus algorithm as implemented in mothur v. 1.26.0 (Schloss et al., 2009) and (b) having a top BLAST match with <90% coverage and <90% identity to a known fungal sequence were also removed from the data set, except those OTUs that have been observed in more than two samples. The OTUs represented by a single sequence (singletons) were also discarded from the data set as suggested by (Tedersoo et al., 2010). OTUs that were detected in the negative control were also removed. The representative sequence from each retained OTU was submitted to BLASTn (Altschul et al., 1997) for comparison against the GenBank nonredundant (NCBI-nr) database. The OTUs with the best match to nonfungal species were removed, and the retained OTUs were used for further analyses.

Statistical analyses

One sample of each replicated pairs were randomly removed (S2.78.2, R1.55, R3.68, R4.45 and R5.32.2) before further analyses. EstimateS v.7.5.2 (Colwell, 2009) was used to calculate the shared number of OTUs in each pair of samples based on the presence/absence form of OTU data set. The occurrence of each OTU in both ridge and snowbed was tested by G-test and ANOVA test in QIIME v. 1.5.0 (Caporaso et al., 2010), with the null hypothesis that each OTU is evenly distributed among the samples. OTU richness and community composition were analyzed using the R software v. 2.15.2 (R Development Core Team, 2009). OTU-accumulation curves and estimates of total species richness of *B. vivipara* root-associated fungi, for both the entire data set and the two vegetation types separately, were calculated as proposed by Ugland et al. (Ugland, Gray, and Ellingsen, 2003) and implemented in R package vegan (Oksanen et al., 2011). Global linear model (GLM) tests were used for

testing the influences of environmental factors and rhizome characteristics on the OTU richness between the ridge and snowbed vegetation types.

The two-dimensional global non-metric multidimensional scaling (GNMDS) (Kruskal, 1964; Minchin, 1987) and detrended correspondence analysis (DCA) (Hill, 1979; Hill and Gauch, 1980) were performed on the presence/absence data, using the package vegan (Oksanen et al., 2011) and MASS (Venables WN and BD, 2002) in R using the Bray-Curtis distances (Bray and Curtis, 1957). The correspondence between axes of DCA and GNMDS was tested by Kendall's rank correlation coefficients. The influence of environmental factors and size and weight of plant rhizome were co-ordinated into the GNMDS ordination as well. Then the correspondence between the effect of these factors (environmental factors and the size and weight of plant rhizome) and GNMDS axes was tested by GLM test.

RESULTS

Obtained data

A total of 191099 sequences were obtained by 454 sequencing, and 151642 sequences were retained after filtering. Using a 97% sequence similarity cut-off, the sequences clustered into 1172 OTUs. Fifty-six of the OTUs were identified as chimeras and removed from the dataset. Moreover, singleton OTUs (298), OTUs appearing in the negative control (6), and OTUs that had best Blast match against non-fungal sequences (11) were removed from the dataset, leaving 801 non-singleton OTUs for further analyses. The five samples that were run in parallel as methodological replicates were more similar to each other in OTU composition compared to between-sample comparisons (Fig. S2, Table S2).

Fungal richness

The level-off by the accumulation curve of fungal OTU richness (Fig. 4a) and estimates of total OTU richness (Fig. 4c) indicate that a large part of the fungal diversity in the *B. vivipara* root systems in the sampling area was detected. No significant difference in observed or estimated fungal OTU richness was found between the snowbed and ridge vegetation types (Fig. 4b). On average, 88 OTUs appeared in each root system, ranging from 13 to 137 (Fig. S3). In samples from ridge, there were on average 83 OTUs per sample, ranging from 13 to 132; in snowbed, the average number of OTUs per sample was 93, ranging from 59 to 137. In a GLM analysis, no significant relationships were found between OTU richness per root system and explanatory variables, including C and N content, vegetation type and rhizome size. The OTU richness was slightly correlated with P concentration across all the samples (Table S3), which indicates that P may play a role in the growth of these root associated fungi.

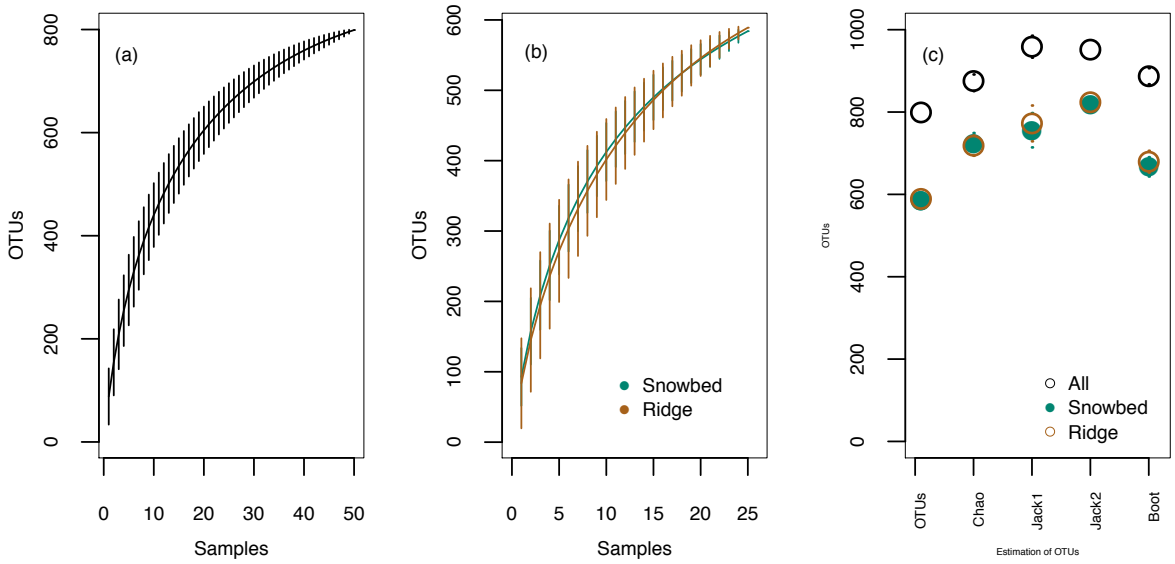

Fig. 4 Accumulation curves of fungal OTU richness for (a) all the sampled *Bistorta vivipara* plant roots, (b) the ridge and snowbed samples separately, as well as (c) observed and estimated total OTU richness using four different richness estimates.

Taxonomic composition

In Table 1, the taxonomic distribution of the detected fungal OTUs is summarized. Most OTUs (66.5%) belonged to Ascomycota and the order Helotiales (52.7%). Only 25.4% of the OTUs were Basidiomycetes and most of these (23.2%) were Agaricomycetes. Other fungal groups, including Glomeromycota, Zygomycota and Chytridiomycota, only constituted 1.9% of the OTUs. When summarizing the number of reads across taxonomy (Table 1), it turned out that most reads were taxonomically affiliated with Basidomycota (65.7%) and the class Agaricomycetes (64.7%). Notably, while Russulales only accounted for 2.6% of the OTUs, as much as 26.9% of the obtained reads belonged to this order. Conversely, the Ascomycete groups were far less abundant when recorded as number of reads. When looking into the presence of OTUs in the two vegetation types, although not significantly different (chi-square tests, $p>0.05$), the ascomycetes and the basidiomycetes were relatively more abundant in the snowbed plots (Table 1). In Table 2, the 15 most common OTUs are listed. As revealed by the G-test, some of the OTUs were significantly affiliated with one of the two vegetation types (Table 3, ANOVA test in Table S4).

Table 1. Summary of the distribution of operational taxonomic units (OTUs) and sequences of fungal lineages found in the root systems of *Bistorta vivipara*. The first two columns show the distribution of OTUs and sequences over all samples, while the last four show the distribution in the ridge and snowbed vegetation.

Taxonomic group	**Total**		**Snowbed**		**Ridge**	
	% OTUs	**% reads**	**% OTUs**	**% reads**	**% OTUs**	**% reads**
Ascomycota	66.47	32.47	33.66	16.24	32.8	16.23
Leotiomycetes	54.54	27.75	26.93	12.82	27.61	14.94
Helotiales	52.75	26.63	26.18	12.46	26.57	14.17
Rhytismatales	1.02	0.97	0.52	0.33	0.5	0.64
Eurotiomycetes	1.27	0.13	0.61	0.05	0.66	0.08
Chaetothyriales	1.16	0.11	0.59	0.05	0.57	0.06
Dothideomycetes	1.18	1.97	0.79	1.68	0.39	0.29
Sordariomycetes	1.13	0.37	0.5	0.29	0.64	0.08
Basidiomycota	25.43	65.72	14.95	37.72	10.48	28
Agaricomycetes	23.21	64.67	13.61	36.74	9.6	27.93
Agaricales	9.46	19.61	5.94	12.99	3.52	6.62
Thelephorales	5.13	12.74	3.99	7.06	1.13	5.68
Sebacinales	2.99	3.76	0.79	1.2	2.2	2.56
Russulales	2.59	26.91	1.52	14.85	1.07	12.06
Tremellomycetes	1.13	0.77	0.64	0.71	0.5	0.05
Other fungal divisions	2.75	0.59	1.43	0.3	1.31	0.29
Glomeromycota	0.98	0.35	0.48	0.14	0.5	0.22
Fungi spp.	5.35	1.22	2.54	0.54	2.81	0.68

Table 2. The 15 most common OTUs. Top hit gives the best match of the representative sequences to NCBI GenBank; OTU ID gives the ID of the OTUs; Top hit in GenBank gives the best match when blasted against NCBI GenBank, with the accession in bracket gives the accession number. Cov (stands for Query coverage) gives the percentage of sequence match against the top hit in GenBank. Iden (stands for Identity) gives the sequence similarity to the top hit. N_A gives the number of samples in which the OTU were observed across all samples. N_R in ridge, and N_S in snowbed. R_A(%) gives the percentage of the number of reads clustered as the OTU across all the samples, and R_R(%) in ridge R_S(%) in snowbed. Only one OTU was found in all 50 samples, with the best match to *Articulospora*. *Russula* sp. was the most abundant OUT found, with 24.6% of all the OTUs.

OTU ID	Top hit in GenBank (accession)	Cov	Iden	N_A	N_R	N_S	R_A(%)	R_R(%)	R_S(%)
1089	*Articulospora* sp.(JN995644)	95	99	50	25	25	5.77	3.2	2.6
444	*Helotiales sp.* (AB598104)[1]	96	98	43	25	18	2.09	1.6	0.49
858	*Meliniomyces* sp. (HQ157926)	95	99	41	20	21	1.02	0.18	0.84
547	*Articulospora* sp. (EU998923)	95	99	38	15	23	1.16	0.38	0.78
383	*Articulospora* sp. (EU998928)	96	98	37	17	20	1.84	0.65	1.19
452	*Articulospora tetracladia* (EU998923)	95	93	37	16	21	0.21	0.1	0.11
918	Helotiales sp. (EU998923)[2]	95	96	37	19	18	0.98	0.5	0.48
1059	*Gyoerffyella* sp. (EU998923)	95	100	37	13	24	0.34	0.14	0.2
494	*Phialocephala* sp. (JQ272456)	96	92	36	18	18	0.18	0.09	0.09
75	*Russula* sp. (AY061696)	96	99	35	16	19	24.64	11.36	13.28
334	*Cortinarium diasemospermus* (AY061696)	96	100	33	11	22	12.4	2.6	9.8
181	*Phialocephala fortinii* (EU882733)	96	99	32	14	18	0.12	0.05	0.07
376	*Meliniomyces* bicolor (HQ157926)	95	96	29	9	20	0.08	0.01	0.07
1057	Helotiales sp. (HQ157926)[3]	95	98	29	17	12	0.4	0.21	0.18
1062	Helotiales sp. (AB598104)[4]	96	90	29	17	12	0.38	0.22	0.16

Best match at species level: [1]*Leptodontium elatius* (acc.no. JF340290, Cov=95%, Iden=96%), [2]*Phialea strobilina* (acc.no. EF596821, Cov=96%, Iden=88%), [3]*Leptodontium elatius* (acc.no. JF340290, Cov=95%, Iden=96%), [4]*Leptodontium elatius* (acc.no. JF340290, Cov=95%, Iden=90%).

Fungal community composition

The most common OTU (OTU ID 1089 with the best match to an *Articulospora* sp. accession JN995644) appeared in all of the root systems, while a high proportion of the OTUs (20.6%) was detected in only a single root system (Fig. S4). The turnover in species composition between root systems was high: on average only 22 out of the 801 (2.7%) OTUs were shared in pair wise comparisons across all the samples. However, both the GNMDS and DCA ordination analyses based on presence/absence OTU data revealed that the fungal community composition in the two vegetation types were clearly different (Fig. 5 and Fig. S5). The GNMDS ordination axes 1 and 2 were strongly correlated with the corresponding DCA axes 1

and 2 (Kendall's Tau = 0.88 and -0.54, respectively, Fig. S5). As the GNMDS ordination plot shown, the two vegetation types were clearly separated along the first axis (Fig. 5). Moreover, samples originating from the same plot clustered closer (Fig. 5), reflecting a spatial effect. The amount of soil phosphorus (P), nitrogen (N) and carbon (C) showed a strong correlation with the first ordination axis. When testing statistically in GLM analyses, the vegetation types as a factor, together with the soil P, N and C, were significantly related with the first axis (p values < 0.05) (Table 4). Moreover, interaction terms between the vegetation type and C or N, and between C and N, also were significantly related to the first axis (Table 4). The different rhizome size parameters (horizontal length, vertical length and thickness of the rhizome) and the weight of root systems had relatively weak effects on the first axis (Table 4). The second axis was correlated with the interaction between the vegetation type and C or N, together with the interaction between C and N. Notably, the horizontal length of rhizome (RHI) was significantly related with the second axis (Table 4).

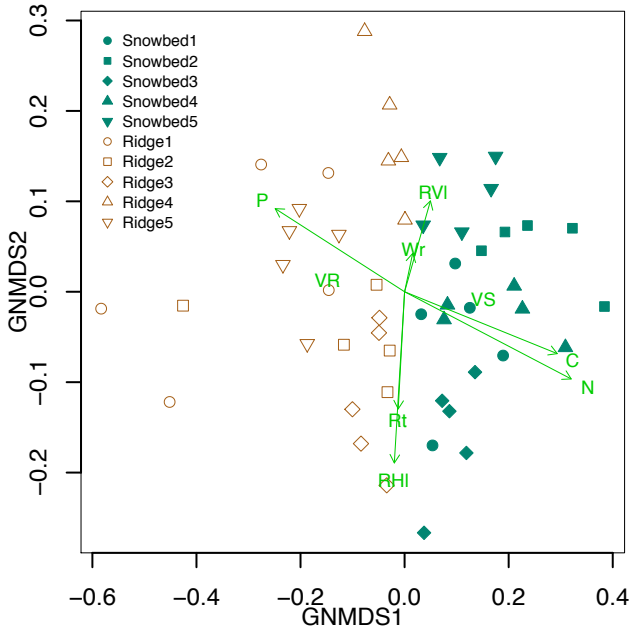

Fig. 5 GNMDS ordination of the fungal communities of all samples according to the presence/absence data. RHI, RVI and Rt stand for the horizontal length, vertical length and thickness of the rhizome; while Wr stands for the weight of the rhizome. VS indicates snowbed, and VR ridge. The samples from the two vegetation types are separated along the first axis, and samples from the same sample plot cluster together to some degree. The environmental factors P, N, C have a strong influence on the ordination, with P having opposite effect to N and C.

Table 3. OTUs that were significantly over- or underrepresented in one of the vegetation types, according to the G-test. The top hit gives the best match when blasted against NCBI GenBank. Query cover gives the percentage of sequence match against the top hit in GenBank, max.id gives the sequence similarity to the top hit and Acc. no. gives the accession number. Prob gives the g-value for an OTU to be non-randomly distributed. Bonferroni gives the Bonferroni-corrected probability for an OTU to be non-randomly distributed across the sample types. Ridge gives the observed number of OTU in the vegetation type, whereas Snowbed lists the number of times a OTU is observed in the snowbed vegetation.

OTU ID	Top hit	Query cover	Max.id	Acc.no.	Prob	Bonferr.	Ridge	Snow-bed
465	*Acephala* sp.	93 %	98 %	GU973749	0.1123	14.3715	12	4
547	*Articulospora tetracladia*	95 %	99 %	EU998923	0.0603	7.7184	15	23
827	*Cadophora finlandica*	95 %	90 %	HQ406816	0.015	1.9169	1	10
984	*Cadophora finlandica*	95 %	91 %	EU557316	0.0011	0.1371	0	10
321	*Cadophora finlandica*	95 %	95 %	HQ406816	0.0462	5.9169	3	12
552	*Cortinarius diasemospermus*	96 %	93 %	JQ724021	0.0004	0.0539	0	11
334	*Cortinarius diasemospermus*	96 %	100 %	JQ724021	0.0103	1.3233	11	22
799	*Cortinarius saniosus*	96 %	99 %	DQ102678	0.0026	0.3362	2	14
1059	*Gyoerffyella* sp.	95 %	100 %	EF093185	0.0028	0.3621	13	24
1107	*Gyoerffyella* sp.	79 %	99 %	EF093184	0.0317	4.0589	1	9
460	Helotiaceae sp.[1]	95 %	94 %	HQ157864	2.54E-08	3.26E-06	0	19
444	Helotiales sp.	96 %	98 %	AB598104	0.0136	1.7466	25	18
667	Helotiales sp.[2]	95 %	97 %	AB598104	0.0001	0.0188	15	1
695	Helotiales sp.[3]	96 %	95 %	AB598104	0.0067	0.8569	11	1
689	*Heterochaete* sp.	19 %	100 %	AF291285	0.0871	11.1465	16	7
35	*Hyaloscypha albohyalina*	89 %	91 %	AB546939	0.0026	0.3362	2	14
752	*Hymenoscyphus monotropae*	96 %	95 %	JX630593	0.0067	0.8569	1	11
1145	*Hymenoscyphus* sp.	95 %	89 %	GU479912	0.0607	7.7736	13	4
436	*Lachnum virgineum*	95 %	97 %	AB481269	0.0143	1.8351	12	2
499	*Lachnum virgineum*	95 %	98 %	JQ272454	0.0317	4.0589	9	1
258	*Meliniomyces bicolor*	95 %	95 %	HQ157926	5.61E-05	0.0072	0	13
376	*Meliniomyces bicolor*	95 %	96 %	HQ157926	0.0172	2.1997	9	20
550	*Meliniomyces bicolor*	77 %	100 %	HQ157926	0.0317	4.0589	1	9
811	*Meliniomyces* sp.	95 %	89 %	EF093175	0.088	11.2591	3	11
681	*Meliniomyces* sp.	95 %	91 %	EF093175	0.0004	0.0539	11	0
1112	*Meliniomyces variabilis*	85 %	100 %	JQ088277	0.0317	4.0589	1	9
413	*Monodictys arctica*	96 %	99 %	EU686521	0.0227	2.9006	3	13
825	*Mycena* sp.	97 %	99 %	HQ157912	0.0303	3.8842	2	11
505	*Phialocephala fortinii*	55 %	99 %	FJ031032	5.61E-05	0.0072	0	13
403	*Phialocephala fortinii*	96 %	91 %	JQ711965	0.0028	0.3621	1	12
848	*Pseudeurotium bakeri*	96 %	88 %	GU934582	4.05E-07	5.18E-05	2	21
451	*Pseudeurotium bakeri*	96 %	88 %	GU934582	5.93E-06	0.0008	0	15
1025	*Rhizoscyphus ericae*	96 %	89 %	JQ711893	0.0067	0.8569	1	11

320	*Rhizoscyphus ericae*	96 %	94 %	JQ711893	0.0067	0.8569	11	1
1051	*Sebacina* sp.	96 %	87 %	JQ711784	0.1125	14.4004	9	2
745	Sebacinales sp.	96 %	93 %	JQ272430	0.0011	0.1371	10	0
784	Sebacinales sp.	96 %	94 %	JQ272430	0.0143	1.8351	12	2
948	Sebacinales sp.	96 %	93 %	JQ272430	0.015	1.9169	10	1
1068	Thelephoraceae sp.	96 %	99 %	U83470	3.45E-05	0.0044	2	18
1128	*Tomentella bryophila*	96 %	98 %	JQ711917	0.0011	0.1371	0	10
1054	*Tomentella* sp.	96 %	98 %	JQ711829	0.015	1.9169	1	10

Best match at species level: [1] *Rhizoscyphus ericae* (acc.no. JQ711893, Cov=96%, Iden=90%), [2]*Leptodontium elatius* (acc.no. JF340290, Cov=95%, Iden=96%), [3]*Leptodontium elatius* (acc.no. JF340290, Cov=96%, Iden=93%)

Table 4. Results from GLM analyses where GNMDS axes one and two (Fig. 3) are related to various environmental factors (C, N and P), plant rhizome characteristics and interaction effects (for example, C*Veg.). V gives the vegetation types (snowbed and ridge). N gives nitrogen in the soil. C gives the carbon content of the soil. P gives the phosphorus of the soil. RHl stands for the horizontal length of the rhizome. RVl gives the vertical length of the rhizome. Rt gives the rhizome thickness and Wr gives the weight of the rhizome. The asterisks give significance level of correspondence of the factor and the axis.

Factor	**GNMDS axis 1**	**GNMDS axis 2**
Vegetation type	7.55e-11 ***	0.365
N	4.55e-05 ***	0.436
C	0.000265 ***	0.556
P	0.00192 **	0.578
C*Veg.	0.00385 **	0.0230 *
N*Veg.	0.00544 **	0.0110 *
N*C	0.00942 **	0.0136 *
RHl	0.668	0.0301 *
P*V	0.12444	0.78
P*C	0.13598	0.319
C*N*V	0.17075	0.215
P*N	0.2408	0.43
RVl	0.364	0.365
Rt	0.776	0.147
Wr	0.781	0.667

DISCUSSION

Fungal community composition

The complex environmental gradient stretching from exposed ridges to snowbeds is highly important for local structuring of vegetation in alpine areas (Fægri, 1967; Dahl, 1986). In contrast to our understanding of the changes in vegetation cover, little is known about how fungal communities change over the ridge-snowbed gradient. The results from this study demonstrate a distinct turnover in the fungal communities associated with roots of *B. vivipara* along the gradient; in the ordination plots there was a clear separation of the samples from ridge and snowbed along the first axis. Several environmental factors vary systematically along the ridge-snowbed gradient, including content of C, N and P, as well as moisture and snow-cover. However, since these factors are highly correlated it is not possible to separate their effects and infer any causal relationships. Similar to the result in the present study, a turnover in ECM fungal community composition was observed along a nutritional gradient in a boreal forest that mirrored the corresponding changes in soil parameters and vegetation (Toljander et al., 2006). Moreover, the soil fungal community composition in boreal peatland was significantly correlated with a litter quality gradient, where the litter chemical composition played a key role in the litter-decomposing fungal community composition (Peltoniemi et al., 2012). Similar trends were also found by Twieg et al. (2009) and Reverchon et al. (2012), where the ECM fungal community composition varied along a soil nutrient gradient. In this study, the plots from ridge and snowbed, respectively, were characterized by significantly different vegetation cover. Changes in vegetation along the ridge and snowbed gradient may also play an important role in the *B. vivipara* root associated fungal community composition. In a volcanic desert, Nara et al. (2006) observed that *Salix* plants, which were pioneer colonizers, provided adjacent late colonizers with compatible ECM fungal symbionts.

In line with the observed turnover in fungal community composition, some of the fungal OTUs were associated with ridges, others with the snowbed vegetation. This could partly be due to adaptations to the different environmental conditions (Reverchon, Ortega-Larrocea, and Perez-Moreno, 2012), but also because of biotrophic interactions (Pickles et al., 2012). Interestingly, several OTUs with taxonomic affinity to Sebacinales showed a distinct

association to the ridges. Sebacinales is an early diverging lineage within Basidiomycota, which can be divided into two main clades (A and B in earlier studies), both having diverse potential to form mycorrhizal associations, ranging from ectomycorrhizae to ericoid and orchid mycorrhizae, but they can also act as endophytes (Weiss et al., 2004; Selosse, Dubois, and Alvarez, 2009). It has been proven that Sebacinales may have a beneficial influence on the host plant growth (Weiss et al., 2011). One might speculate that the Sebacinales fungi have a more profound influence on the host plant growth in the poorer ridge habitat (Reverchon, Ortega-Larrocea, and Perez-Moreno, 2012).

On the other hand, numerous OTUs with taxonomic affinity to the ECM forming *Tomentella* and *Cortinarius* genera were strongly associated with the snowbeds. *Tomentella* species have earlier been found to be the dominant ectomycorrhizal partners of alpine ECM plants like *Kobresia myosuroides* (Muhlmann and Peintner, 2008a), *Salix herbacea* (Muhlmann and Peintner, 2008b) and *B. vivipara* (Muhlmann, Bacher, and Peintner, 2008). Whether the *Tomentella* species are outcompeted at the ridges by e.g. Sebacinales fungi or not adapted to this poorer habitat is unknown. *Cortinarius* species are typically associated with well-decomposed organic matter and humus (Lindahl et al., 2007), and therefore may thrive better in the snowbeds where there is a high amount of organic material. Likewise, OTUs with taxonomic affinity to the dark septate root endophytes, like the *Cadophora finlandica/Meliniomyces* spp. complex and the *Phialocephala fortinii* complex, showed distinct preferences for the snowbeds (with only the exception of one *Meliniomyces* OTU). In line with this result, Summerbell et al. (2005) observed that the root endophyte fungus *Meliniomyces variabilis* was most common in peat bog sites, where humus were better decomposed. Notably, two OTUs with taxonomic affinities to the *Rhizoscyphus ericae* complex showed opposite preferences for ridge and snowbed. Further studies are needed to conclude whether this is due to adaptation to different environments or other factors.

The ordination analyses indicated that additional factors may play a role in structuring the root associated fungal communities. Notably, the horizontal length of the rhizome was significantly associated with the second GNMDS axis. *Bistorta vivipara* is a perennial plant and the size of the rhizome could be positively correlated to the plant age. Hence, *B. vivipara* roots of different age could harbor slightly different fungal communities. In support of this hypothesis, Pickles et al. (2012) observed that host age can affect the structure of ECM

communities. They also observed age-dependent distribution of fungal endophytes in *Panax ginseng* roots.

At a finer scale, there was a tendency for a spatial patterning of the fungal communities, where plant roots from the same plot had a somewhat more similar community composition compared to across plot comparisons. Neighboring plants may share more fungal partners due to belowground vegetative growth between adjacent root systems (Bingham and Simard 2012). Similar findings have also been discussed by Selosse et al. (2006), that a common mycorrhizal network can form when fungal mycelia colonize and link together the roots of two or more plants, even of different plants species. The networks can in turn affect the physiology and ecology of plants by facilitating interplant nutrient exchange (Teste et al., 2009). Moreover, limited spore dispersal may also cause a spatial autocorrelation effect. As Galante et al. (2011) demonstrated, most basidiospores generally fall within a very limited area from the cap.

Although a systematic shift in fungal community composition was observed from ridge to snowbed, there was also a high heterogeneity and a low overlap in number of shared OTUs across the 50 root systems. This supports that a high degree of stochasticity is involved during the assembly of fungal communities (Izzo, Agbowo, and Bruns, 2005; Lekberg et al., 2012; Pickles et al., 2012). This might partly be due to diverse spore dispersal processes of different fungal groups (Bruns, 1995). Other factors leading to high heterogeneity might be variable niche partitioning of ECM fungi (Tedersoo et al., 2003) due to their different enzymatic capabilities (Abuzinadah and Read, 1986; Bruns, 1995), and competitive interactions between different fungal species, like species replacements, co-infections of ectomycorrhizal fungi on single host roots, and rootlet turn-over (Bruns, 1995), which have specially strong influences on ECM fungal community in root tips (Pickles et al., 2012).

Richness and taxonomy

A large and diverse assemblage of root-associated fungi was detected in this study with a total of more than 800 non-singleton OTUs. The perennial life history of *B. vivipara* may allow for a continuous accumulation of species over numerous years. As indicated by the accumulation curves, a large part of the fungal diversity associated with *B. vivipara* roots in the area were recovered. The accumulation curves and the different estimates of total OTU richness together

indicated no distinct differences between the nutrient poor ridge and the snowbed in fungal species richness. Hence, while the species composition changes along the gradient, the species richness does not. Similar diversity patterns were detected when comparing alpine open meadows and willow understory habitats (Becklin, Hertweck, and Jumpponen, 2012) and different parts of a salinity gradient (Mohamed and Martiny, 2011).

Most of the OTUs recovered in this study belonged to the Dikarya (Ascomycota and Basidiomycota). Ascomycota was most diverse when it comes to OTU richness (66.5% of the OTUs) while the basidiomycetes dominated when it comes to proportion of reads (65.7%). This probably reflects their different life strategies. Many of the detected basidiomycetes are high biomass ECM fungi that will yield many reads in high throughput sequence analyses of bulk samples. In contrast, a higher proportion of the ascomycetes probably represent root endophytes or pathogens of less biomass that will end up in relatively fewer reads.

At a lower taxonomic level (order) Helotiales was found to be the most OTU rich group (54.54%) followed by the largely ECM forming basidiomycete orders Agaricales (9.46%), Thelephorales (5.13%), Sebacinales (2.99%) and Russulales (2.59%). This taxonomic distribution is to a large extent in accordance with what was found in studies on ECM fungal community associated with *Kobresia* species (Gao and Yang, 2010) and the rhizosphere fungi colonizing three alpine plant species, *Taraxacum ceratophorum*, *T. officinale*, and *Polemonium viscosum* (Becklin, Hertweck, and Jumpponen, 2012). Notably, the ECM forming Russulales had a very high proportion of reads (26.9%) compared to OTUs (2.59%), which indicates a high biomass.

Among the most common OTUs across all samples were several OTUs with high sequence similarity to *Articulospora* spp. *Articulospora* is aquatic hyphomycetes forming characteristic spores spreading through water (Quilliam and Jones, 2010; Seena et al., 2012). Aquatic hyphomycetes are also known as ingoldian fungi (Ingold, 1942), many of which seem to spend parts of their life cycle as root endophytes (Selosse, Vohnik, and Chauvet, 2008). Helotiales spp. and *Meliniomyces* spp. were also widely detected across the samples. One possible reason for that could be that these fungi have diverse potential associations with plant roots. On one hand, some species from Helotiales and *Meliniomyces* belong to dark septate root endophytes (Ohtaka and Narisawa, 2008; Upson et al., 2009), and therefore have the abilities to establish endophytic association with host plants. On the other hand,

Meliniomyces species were suggested to be able to establish both endophyta and mycorrhiza (Hambleton and Sigler, 2005), whereas Helotiales species were observed as typical ericoid mycorrhizal fungi (Walker et al., 2011).

This study demonstrates that the fungal communities associated with roots of the ECM forming plant *B. vivipara* change systematically along a ridge-snowbed gradient, which is a very important environmental gradient in alpine areas, and that various fungal groups are associated with the different environmental conditions. Moreover, the study supports the view that there is a high and largely uncharacterized diversity of different fungal groups in alpine regions, many of which do not form macroscopic fruit bodies.

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SUPPLEMENTARY

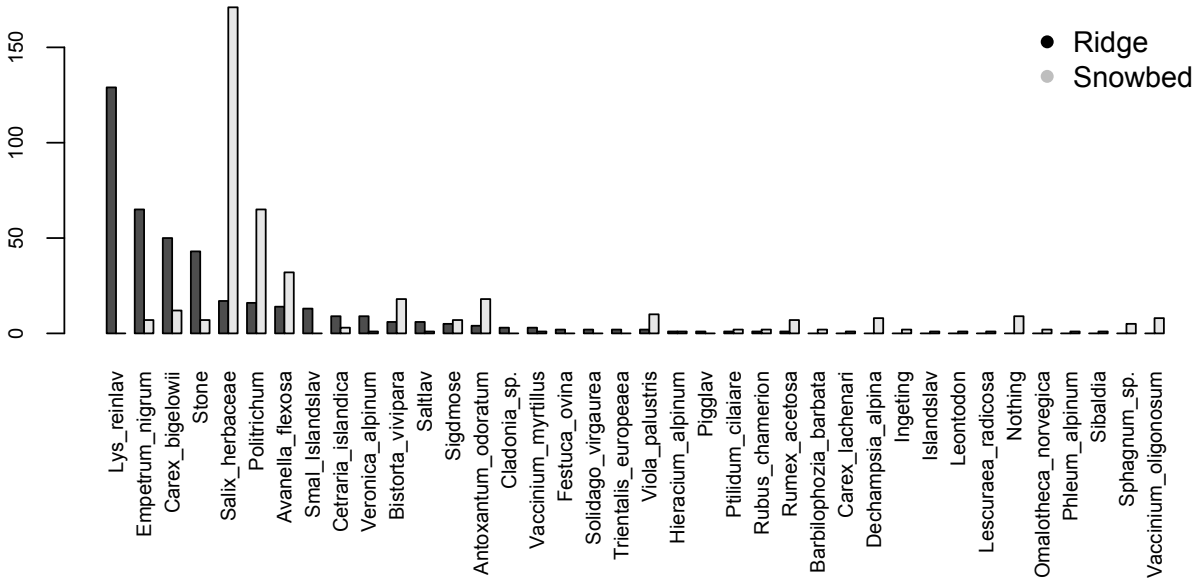

Fig. S1 The vegetation cover in the sampled plots in ridge (black bars) and snowbed (grey bars).

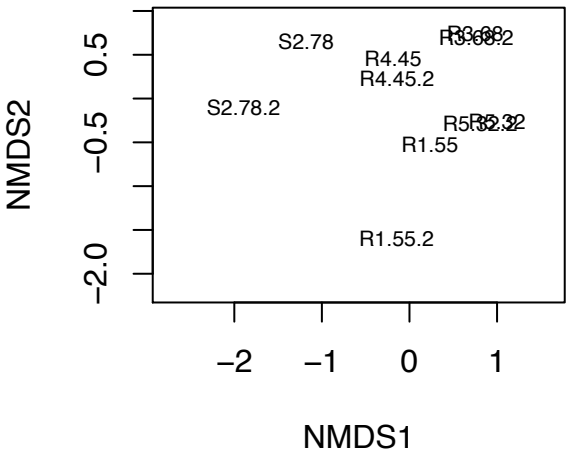

Fig. S2 GNMDS ordination plot of the replicated samples.

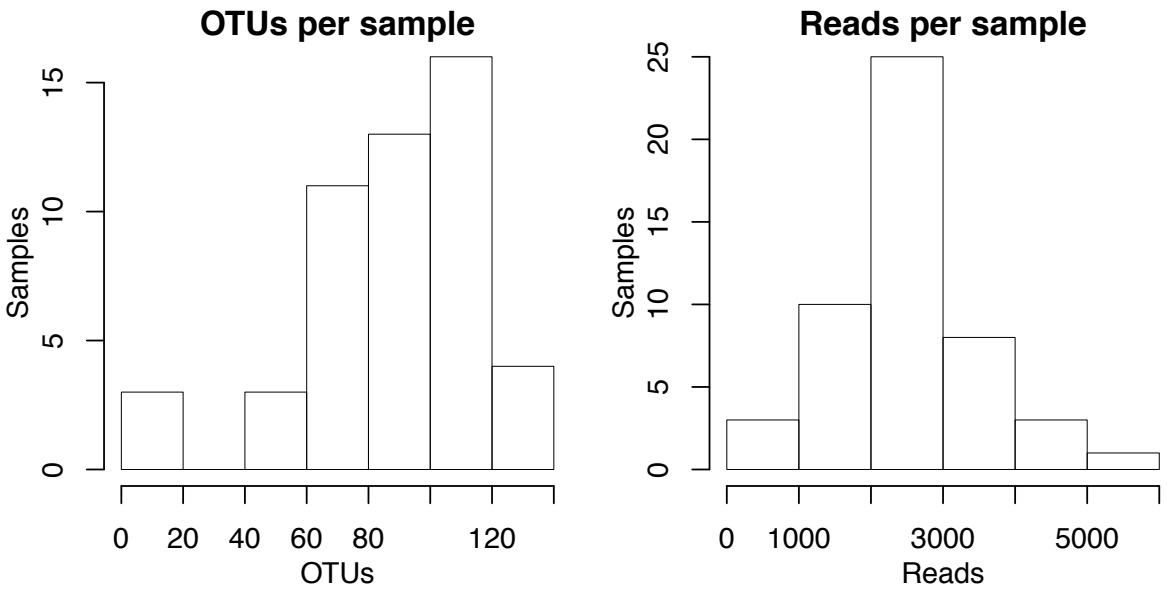

Fig. S3 Comparision of number of *Bistorta vivipara* plant root samples with the number of fungal OTUs and fungal reads.

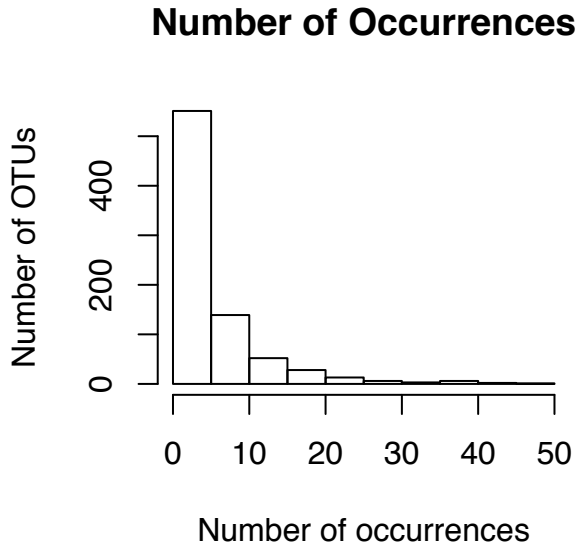

Fig. S4 Number of occurrence of fungal OTUs in the samples.

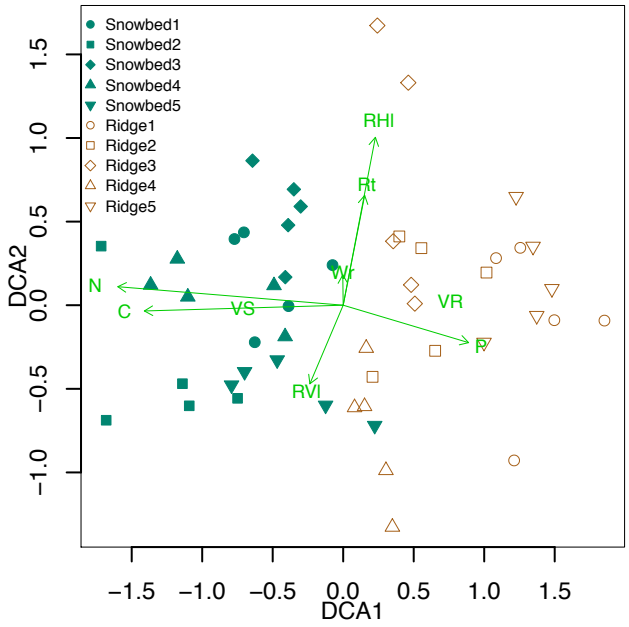

Fig. S5 The DCA ordination of the fungal communities of the *Bistorta vivipara* plant root samples.

Table S1. How DNA samples were tagged by pyrotags. Numbers 1-56 were PCR number. Each four samples shared the same tag, shown with tag name and sequence in table. Then each culumn of samples was collected into a new eppendorf tube, shown with Epp. Tube number 1-4. Thus samples in each tube have different tags on. These four tubes were sent for 454 sequencing

Epp. Tube 1	Epp. Tube 2	Epp. Tube 3	Epp. Tube 4	Pyrotags used	
1	2	3	4	TCMID31	AGCGTCGTCT
5	6	7	8	TCMID32	AGTACGCTAT
9	10	11	12	TCMID33	ATAGAGTACT
13	14	15	16	TCMID34	CACGCTACGT
17	18	19	20	TCMID35	CAGTAGACGT
21	22	23	24	TCMID36	CGACGTGACT
25	26	27	28	TCMID37	TACACACACT
29	30	31	32	TCMID38	TACACGTGAT
33	34	35	36	TCMID39	TACAGATCGT
37	38	39	40	TCMID40	TACGCTGTCT
41	42	43	44	TCMID41	TAGTGTAGAT
45	46	47	48	TCMID42	TCGATCACGT
49	50	51	52	TCMID43	TCGCACTAGT
53	54	55	56	TCMID44	TCTAGCGACT

Table S2. The total number of sequences and OTUs in each replicated samples. The samples S2.78.2 had relatively low number of sequences, and the R1.55.2 had very low number of OTUs. The diferrences within S2.78 pair, and R1.55 pair are bigger than other pairs.

	S2.78	S2.78.2	R1.55	R1.55.2	R3.68	R3.68.2	R4.45	R4.45.2	R5.32	R5.32.2
Total number of sequences	3380	118	965	57	1685	3433	1193	1480	3615	5057
Total number of OTUs	70	21	53	12	108	109	62	67	74	68

Table S3. The GLM analyses testing the effects of environmental variables on the number of OTUs in each sample.

Variable factors	Number of OTUs in each sample
Vegetation type	0.252
P	0.0165 *
N	0.177
C	0.157
Wr	0.716
RVl	0.762
RHl	0.442
Rt	0.507

Table S4. The ANOVA test of the OTUs in the samples.

OTU	prob	Bonferroni_corrected	FDR_corrected	1_mean	2_mean
460	3.22E-05	0.004154	0.004154	0.008405	0
848	0.000173	0.022355	0.011177	0.004661	7.53E-05
451	0.000507	0.065395	0.021798	0.000463	0
334	0.000822	0.106034	0.026508	0.165692	0.043685
667	0.001203	0.155209	0.031042	2.15E-05	0.000815
403	0.001446	0.186482	0.03108	0.000489	1.96E-05
505	0.001767	0.227924	0.032561	0.000594	0
444	0.002182	0.281512	0.035189	0.010521	0.052968
376	0.002233	0.288104	0.032012	0.001414	0.000275
1025	0.003883	0.500921	0.050092	0.000323	2.28E-05
552	0.004584	0.591373	0.053761	0.000913	0

681	0.005505	0.710141	0.059178	0	0.001457
984	0.007414	0.956402	0.073569	0.000383	0
550	0.010113	1.304625	0.093187	0.000281	2.70E-05
1125	0.012351	1.593257	0.106217	0.000189	0.001673
1128	0.018253	2.354617	0.147164	0.026431	0
35	0.02126	2.742584	0.161328	0.000883	8.99E-05
1149	0.024187	3.120186	0.173344	2.92E-05	0.000297
576	0.024513	3.16215	0.166429	0.000303	0.00464
1107	0.025352	3.270459	0.163523	0.000321	2.50E-05
465	0.028763	3.710483	0.17669	0.000256	0.001852
653	0.033751	4.353925	0.197906	0.000585	0.003441
1054	0.033907	4.373965	0.190172	0.018317	1.65E-05
948	0.036832	4.751274	0.19797	1.73E-05	0.011531
745	0.039893	5.146182	0.205847	0	0.011169
793	0.046668	6.020125	0.231543	0.000201	0.000593
320	0.053	6.837048	0.253224	0.000177	0.003236
689	0.053794	6.939485	0.247839	0.001218	0.01445
709	0.054195	6.991191	0.241076	7.18E-05	0.001037
377	0.05513	7.11171	0.237057	7.52E-05	0.000231
655	0.05594	7.216281	0.232783	0.000545	0.004229
436	0.058788	7.58369	0.23699	0.000321	0.041797
1089	0.061949	7.99148	0.242166	0.049566	0.080656
1145	0.065591	8.461249	0.24886	0.000201	0.000523
929	0.06761	8.721642	0.24919	0.000851	0.000212
825	0.069831	9.008213	0.250228	0.010682	0.001728
490	0.073766	9.51577	0.257183	0.000687	0.000177
695	0.074597	9.622983	0.253236	0.001115	0.004819
1135	0.075187	9.699059	0.248694	0.001764	0.00055
1112	0.078372	10.10995	0.252749	0.001442	0.000232
672	0.079016	10.19301	0.24861	0.000103	0.00051
652	0.079262	10.22474	0.243446	0.000122	0.001515
106	0.085383	11.01443	0.256149	9.78E-05	0.000321
499	0.089005	11.4817	0.260948	5.01E-05	0.011808
1119	0.093093	12.00896	0.266866	7.32E-05	0.001403
258	0.100335	12.94327	0.281375	0.003218	0
205	0.10135	13.07421	0.278175	0.00131	0.012587
326	0.108817	14.03743	0.292446	0.011653	0.041915
169	0.11284	14.55635	0.297068	0.001846	0.004067
799	0.112956	14.57137	0.291427	0.003884	3.79E-05
752	0.137877	17.78612	0.348747	0.000821	0.000235
1059	0.139801	18.03434	0.346814	0.004004	0.002194
986	0.14176	18.28704	0.345039	0.00015	0.000583
824	0.143494	18.51074	0.342791	0.000204	0.001392
962	0.147954	19.08607	0.34702	0.000679	0.000234
659	0.153699	19.82713	0.354056	0.000238	0.000764
703	0.156716	20.2163	0.354672	0.000238	6.14E-05

244	0.157464	20.31286	0.350222	0.00021	5.50E-05
321	0.159216	20.53887	0.348116	0.004879	0.000228
603	0.174225	22.475	0.374583	0.013982	0.001301
375	0.181322	23.39053	0.383451	0.003977	0.001117
486	0.185002	23.86532	0.384924	0.000506	0.000194
596	0.185942	23.98651	0.380738	0.000579	0.000122
383	0.186884	24.10801	0.376688	0.026816	0.012323
858	0.188652	24.33613	0.374402	0.016174	0.006617
132	0.197741	25.50864	0.386495	3.56E-05	0.000829
827	0.202232	26.08794	0.389372	0.001013	1.94E-05
1048	0.2025	26.12249	0.384154	9.13E-05	0.000561
770	0.205133	26.46214	0.383509	0.002399	0.000609
784	0.205533	26.51378	0.378768	0.0122	0.031965
183	0.206606	26.65213	0.375382	0.000102	0.000413
575	0.215353	27.78056	0.385841	0.003495	0.000595
276	0.219569	28.3244	0.388005	0.000254	0.000107
434	0.22581	29.1295	0.393642	0.000833	0.000242
1002	0.228283	29.44854	0.392647	0.000103	0.000915
189	0.229593	29.61755	0.389705	0.002913	0.00075
413	0.231495	29.86287	0.387829	0.005157	0.000278
261	0.245154	31.6249	0.405447	0.000884	0.000211
284	0.250956	32.37332	0.409789	0.000622	0.002702
240	0.262108	33.8119	0.422649	0.002056	0.000229
1051	0.271733	35.05351	0.432759	0.000424	0.001706
1057	0.287868	37.13497	0.452865	0.003638	0.006846
351	0.302804	39.06176	0.470624	9.22E-05	0.000209
449	0.305259	39.37846	0.468791	0.000246	0.005654
951	0.308239	39.76282	0.467798	0.000157	0.000365
1062	0.3083	39.77065	0.462449	0.003359	0.005385
1045	0.310621	40.07014	0.460576	0.000565	0.001561
530	0.313017	40.3792	0.458854	0.000983	0.000332
718	0.339648	43.81462	0.492299	0.000247	0.000124
544	0.359845	46.42	0.515778	0.003062	0.006094
260	0.364082	46.96652	0.516116	0.000168	0.000505
900	0.365302	47.12392	0.512217	0.000438	0.003637
510	0.368265	47.50625	0.51082	0.000172	0.000753
1116	0.373751	48.21393	0.512914	0.001018	0.000561
547	0.397697	51.30287	0.54003	0.016055	0.008143
1127	0.407378	52.5518	0.547415	0.000617	0.000398
669	0.418749	54.01858	0.556893	0.00028	0.000134
61	0.423421	54.62134	0.557361	0.003049	0.005104
152	0.442024	57.02107	0.57597	0.00621	0.00378
664	0.448726	57.88571	0.578857	0.000102	0.000196
857	0.464895	59.97148	0.593777	0.016235	0.008304
494	0.495508	63.92049	0.626672	0.001873	0.002466
24	0.501283	64.66548	0.62782	0.000812	0.001331

1090	0.502793	64.86029	0.623657	0.006628	0.003241
918	0.504985	65.14305	0.62041	0.007707	0.011095
435	0.52264	67.42051	0.636043	0.027098	0.012825
21	0.52293	67.45792	0.630448	0.00056	0.000394
450	0.574146	74.06479	0.685785	0.002324	0.003426
402	0.598627	77.22293	0.708467	0.000402	0.000278
181	0.603317	77.82791	0.707526	0.001533	0.001076
366	0.611022	78.82188	0.710107	0.000815	0.001123
593	0.636813	82.14883	0.733472	0.000133	0.000191
100	0.656976	84.74993	0.749999	0.000789	0.001313
1137	0.659122	85.02674	0.745849	0.000488	0.00066
287	0.677359	87.37928	0.75982	0.001658	0.001317
453	0.727902	93.89934	0.809477	0.002217	0.003039
310	0.733264	94.59111	0.808471	0.006656	0.009574
211	0.744725	96.06948	0.814148	0.000157	0.000202
976	0.79698	102.8104	0.863953	0.000252	0.000305
45	0.813143	104.8954	0.874128	0.001492	0.001732
836	0.831418	107.253	0.886388	0.003901	0.004854
559	0.863006	111.3277	0.912522	0.001174	0.001351
1068	0.865289	111.6223	0.907499	0.047472	0.054732
75	0.889237	114.7116	0.925093	0.205084	0.214998
452	0.901302	116.268	0.930144	0.002518	0.002418
811	0.908752	117.2291	0.930389	0.00137	0.001247
919	0.944948	121.8983	0.959829	0.001675	0.001756
81	0.958182	123.6055	0.965668	0.000188	0.000197
892	0.975279	125.8109	0.975279	0.000212	0.000217